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Properties of *Escherichia coli* Mutants Altered in Calcium/Proton Antiport Activity

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Mutants sensitive to growth inhibition by $CaCl_2$ were found to have alterations in calcium uptake in everted membrane vesicles. These mutations map at different loci on the *Escherichia coli* chromosome. A mutation at the *calA* locus results in vesicles which have two- to threefold higher levels of uptake activity than vesicles from wild-type cells. The *calA* mutation is phenotypically expressed as increased sensitivity to $CaCl_2$ in a strain also harboring a mutation in the *corA* locus, which is involved in Mg^{2+} transport. The *calA* locus maps very close to *purA* and *cycA* at about min 97. The *calB* mutation results both in sensitivity to $CaCl_2$ at pH 5.6 and in vesicles with diminished calcium transport capability. The CalB phenotype is also expressed only in a *corA* genetic background; the *calB* locus appears to map very near, yet separately from, the *calA* locus. When the cor^+ allele is present, *calA* and *calB* mutations still result in a defect in calcium transport in vesicles. In addition, both *calC* and *calD* mutations result in vesicles with impaired calcium transport activity. *calC* is cotransducible with *kdp* and *nagA*, whereas *calD* is cotransducible with *proC*.

It is now apparent that bacterial cells, as well as mammalian cells, lower their intracellular concentrations of calcium ions by active extrusion (28, 30). As elegantly put forth by Mitchell (19) and recently reviewed by Rosen and Kashket (25), transport systems which are coupled to energy supplied by previously formed electrochemical gradients can utilize one of three basic mechanisms: symport, uniport, or antiport. In *Escherichia coli* calcium is extruded by such a mechanism (26). A similar system has been found in *Azotobacter vinelandii* (4); an analogous antiport system using Na^+ in place of H^+ exists in *Halobacterium halobium* (3). Both calcium/proton and calcium/sodium antiport systems have been demonstrated in mitochondria (9; W. Dubinsky, M. A. Kandrach, and E. Racker, Fed. Proc. **38**:248, 1979). On the other hand, Harold and co-workers have shown the existence of a calcium extrusion system in *Streptococcus faecalis* linked directly to the hydrolysis of ATP (13).

We previously showed that respiring everted vesicles of *E. coli* are able to accumulate Ca^{2+}, presumably by the same system that is responsible for calcium extrusion from whole cells (32). A proton antiport mechanism was suggested by experiments in which an artificially imposed transmembrane pH gradient, acid interior, was able to drive calcium uptake into everted vesicles (33). Nigericin, in the presence of potassium, inhibited this uptake, demonstrating a requirement for a pH gradient. The massive accumulation of calcium within the vesicles was dependent on the presence of phosphate, which probably co-precipitated with the calcium inside the vesicles.

Recently, utilizing an assay based on the ability of calcium to affect quinacrine fluorescence (a monitor of the transmembrane pH gradient), we showed that the calcium/proton antiport system (Ca^{2+}/H^+ [or CHA] system) of everted vesicles displays complex kinetics, being cooperative with each of its substrates, Ca^{2+}, Mn^{2+}, Sr^{2+}, and Ba^{2+} (6, 7). In addition, La^{3+} and Mg^{2+} were shown to be allosteric inhibitors of the Ca^{2+}/H^+ system (7). The Ca^{2+}/H^+ system showed a requirement for a membrane potential, suggesting that the stoichiometry is $H^+:Ca^{2+} > 2$ (7).

To elucidate the molecular mechanisms underlying calcium/proton antiport activity, we have undertaken a study of mutants which are defective in this activity. Mutants that appear to be altered in calcium/proton antiport activity occur in at least four separate genes. A preliminary report of this material has been presented (24).

MATERIALS AND METHODS

Growth media. For preparation of everted vesi-

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cles, cells were grown in a basal salts medium (31) supplemented with 0.5% glycerol, 10 μg of thiamine per ml, 0.1 mg of required amino acids per ml, or 40 μg of adenine or uracil, per ml, as required. For solid media, agar was added to a concentration of 1.5%.

For determination of calcium sensitivity, medium E (8), containing 0.5% glycerol and adjusted to pH 7.8, was used with either 25 mM or 50 mM $CaCl_2$. Calcium sensitivity at pH 5.6 was determined on medium H plates containing 0.5% glycerol and 50 mM $CaCl_2$. Medium H contained per liter: 10.4 g of morpholinopropane sulfonate, 4.08 g of NaCl, 1.07 g of NH_4Cl, 0.426 g of Na_2SO_4, 0.82 g of $MgSO_4$, and 5 g of peptone (Difco), and was adjusted to pH 5.6 with KOH.

For matings and transduction experiments, cells were propagated in L broth (18).

Isolation of mutants. Calcium-sensitive mutants were isolated from several wild-type strains after mutagenesis by ethyl methane sulfonate by the method outlined by Miller (18). A mutagenized culture of a wild-type strain was inoculated into 10 growth tubes containing 5 ml of L broth each. After overnight incubation, a portion of each was inoculated into flasks containing 10 ml of medium E containing 0.5% glycerol and 50 mM $CaCl_2$. After several generations, 1,000 U of penicillin (sodium salt) per ml was added and incubation was continued for 2 h. Survivors (4×10^4 to 10^5 per ml) were plated on medium E plates containing 0.5% glycerol and 5 mM $CaCl_2$. Small colonies arising after several days were judged to be potential calcium-sensitive derivatives. Those colonies which grew on medium E but not on medium E containing 50 mM $CaCl_2$ were purified and retained. Only one mutant from each selection was saved, so the mutants studied were not sibling isolates. In some cases, calcium-sensitive isolates were obtained directly from penicillin selection by plating onto medium E plates. Strain 2561 was isolated from AW574 after mutagenesis by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (1) and penicillin selection in the presence of 25 mM $CaCl_2$.

Nomenclature. We have chosen the mnemonic *cal* to describe mutations that appear to specifically affect calcium transport in everted vesicles. In the case of *calC* and *calD*, it is clear that these mutations represent different gene products. Although CalA and CalB phenotypes could represent mutations in the same gene, several experiments described below suggested that *calA* and *calB* were separate genetic defects.

The strains used or isolated in this study are listed in Table 1.

Genetic techniques. Preparation of P1*kc* and transduction techniques were essentially as described by Miller (18). Matings between Hfr and F^- cells were performed by mixing 0.1 ml of log-phase Hfr grown in L broth with 0.9 ml of recipient also grown to midlog phase in L broth. After 30 min the mating mixture was diluted 10-fold into basal salts medium containing 0.1 mg of streptomycin sulfate per ml, followed by two cycles of centrifugation and washing. Appropriate dilutions of the washed mating mixture were plated onto selective media. Matings between F' strains and F^- recipients were performed as above, except that F' strains were counterselected by lack of a required amino acid.

Calcium sensitivity in recombinants obtained by mating or transduction was determined in either of two ways: for mapping *calC* and *calD*, calcium-resistant or calcium-sensitive clones could be distinguished by replica plating onto medium E plates containing 50 mM $CaCl_2$; for mapping of *calA*, it was found that single colonies had to be isolated on medium E plates containing 25 mM $CaCl_2$ to distinguish between sensitive and resistant clones.

All initial mutants studied were able to grow in the presence of 5 mM $CaCl_2$, and so P1*kc* could be propagated on them. P1*kc* could not, however, be grown on *calA calB corA* strains presumably because of the calcium requirement for phage adsorption.

Vesicle preparation and assay of $^{45}Ca^{2+}$ uptake. Preparation of everted membrane vesicles was performed as described elsewhere (7, 27). For determination of calcium/proton antiport activity by the quinacrine fluorescence assay, the lysis buffer contained 0.14 M choline-Cl instead of KCl. NADH-driven $^{45}Ca^{2+}$ uptake was assayed as previously described (27), except that the assay buffer consisted of 10 mM Tris-hydrochloride, containing 0.14 M KCl and 5 mM potassium phosphate, adjusted to pH 8.0 with HCl.

Assay of Ca^{2+}/H^+ activity by quinacrine fluorescence. The ability of $CaCl_2$ to affect the transmembrane pH gradient was measured by the enhancement of quenched quinacrine fluorescence as described previously (6, 7).

Protein determination. Vesicle and whole cell protein was determined by a micromodification of the method of Lowry et al. (16), using bovine serum albumin as a standard. In some cases protein was measured by the dye-binding technique of Bradford (5), using bovine serum globulin as the standard.

Chemicals and isotopes. $^{45}CaCl_2$ (1.3 Ci/mmol) was purchased from New England Nuclear Corp., as were L-[^{3}H]lysine and L-[^{3}H]glutamate. All other chemicals were reagent grade and were purchased from commercial sources. The ionophore A23187 was the generous gift of Robert Hamill of Eli Lilly & Co.

RESULTS

Growth properties of mutants. Mutants of *E. coli* defective in calcium/proton antiport activity might be expected to be sensitive to growth inhibition by $CaCl_2$, which would accumulate to inhibitory levels within the cytoplasm of such mutants. Calcium-sensitive mutants were isolated from either strain KBT001 or RB202 by penicillin selection after mutagenesis by ethyl methane sulfonate as described in Materials and Methods. A number of mutants which did not grow in the presence of 50 mM $CaCl_2$ were obtained, and two, RB063 and RB073, were studied more closely. In addition, strain 2561 was isolated from a mutagenized culture of AW574, followed by penicillin selection. The growth properties of these strains are summarized in Table 2.

RB063 and RB073 formed small colonies on medium E plates containing 25 mM $CaCl_2$ after at least 48 h of incubation, but no growth was observed at 50 mM $CaCl_2$. Strain 2561 was unable to form colonies on 25 mM $CaCl_2$ even after

TABLE 1. *Bacterial strains*

Bacterial strains[a]	Genotype[b,c]	Source (reference)
AW574	*thr leu his lac xyl rpsL*	J. Adler
KBT001	*lysA metE purE trp leu proC ara lac rpsL*	R. J. Kadner (12)
RB202	*metB argH trp leu purE proC cycA ara lac rpsL*	This study
χ316	*cycA supE*	B. Bachmann
MP2	*leu his lac corA rpoB rpsL*	J. Lusk (23)
G19	*gltA poaA purE his lac rpsL*	B. Bachmann
AB1321	*proA aroA his galK lac xyl mtl tsx*	B. Bachmann
FRAG-5	*kdpABC rha gal lac*	W. Epstein (10)
TK2240	*trkD1 trkA405 nagA lac*	W. Epstein (10)
MA96	*ilv met his argH rpsL*	W. K. Maas
2561	As AW574 but *corA calA*	This study
RB111	As 2561 but *calB*	NTG-induced from 2561[d]
RB107	As 2561 but *leu*$^+$ *thr*$^+$ *metB calA calB*	JC12 × RB111
RB105	As 2561 but *leu*$^+$ *metB*	JC12 × 2561
RB106	As RB105 but *argH metB*$^+$	P1 transduction via MA96
RB120	As RB106 but *metB*	Spontaneous *metB*
RB121	As RB106 but *metE*	Spontaneous *metE*
RB134	*thr his metA argH lac rpsL calA corA*	AB1927 × 2561
RB146	As RB134 but *argH*$^+$ *metA*$^+$ *calA*$^+$	Ra2 × RB134
RB158	*pyrB his lac rpsL recA calA calB corA*	This study
RB160	*pyrB purA his lac rpsL calA corA*	This study
RB165	As RB121 but *corA*$^+$ *metE*$^+$ *ilv*	P1 transduction via MA96
RB205	As RB197 but *met*$^+$ *cor*$^+$	KL209 × RB107
RB063	As RB202 but *calC*	EMS-induced[d]
RB073	As KBT001 but *calD*	EMS-induced
RB199	As RB063 but *pyrD*	Spontaneous *pyrD*
RB214	As RB199 but *aroA*	P1 transduction via AB1321
RB228	As RB214 but *galE aro*$^+$	PL2 × RB214
RB241	As RB228 but *gltA gal*$^+$	P1 transduction via G19
RB252	As RB241 but *kdpABC5 glt*$^+$	P1 transduction via FRAG-5
RB257	As RB252 but *nagA kdp*$^+$	P1 transduction via TK2240
Ra2	Hfr *supE*	R. J. Kadner
PL2	Hfr *galE*	L. Leive
JC12	Hfr *metB purB,C*	W. K. Maas
AB1927	Hfr *metA argH*	B. Bachmann
KLF17/KL132	F'117 *purA*$^+$ *purB*$^+$/*thr leu his pyrB pro thyA recA xyl malA ara gal lac rpsL*	B. Bachmann
KLF19/KL132	F'119 *pyrB*$^+$/*thr leu his pyrB pro thyA recA xyl malA ara gal lac rpsL*	B. Bachmann
KLF26/KL181	F'126 *nadA*$^+$ *rac*$^+$/*pyrD his trp recA mtl xyl malA galK rpsL*	B. Bachmann (14)
F152/KL253	F'152 *lip*$^+$ *gal*$^+$/*pyrD his trp tyrA recA mtl malA galK rpsL*	B. Bachmann (14)

[a] The first three strains listed are the parental strains used in the isolation of the various *cal* mutations.

[b] All strains required thiamine for growth and are F unless otherwise stated.

[c] The properties and origins of transfer of Hfr strains not listed in Table 1 are described by Low (15). AB1927 and JC12 have origins and directions of transfer similar to KL14; PL2 has the origin of Hfr Hayes.

[d] Abbreviations: NTG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; EMS, ethyl methane sulfonate.

prolonged incubation. As shown below, strain 2561 harbors both *corA* and *calA* defects. Park et al. (23) reported that a mutant defective in the *corA* gene, which is involved in magnesium transport, was sensitive to growth inhibition by 50 mM $CaCl_2$ but resistant to 25 mM (Table 2). The defects in RB063 and RB073 map at different loci and have been designated as *calC* and *calD*, respectively.

Calcium sensitivity in strains 2561, RB063, and RB073 can be eliminated by lowering the pH of the medium to pH 5.6 (Table 2). It was possible to select from strain 2561 a mutant (RB111) sensitive to 50 mM $CaCl_2$ at pH 5.6. The mutation resulting in sensitivity to $CaCl_2$ at pH 5.6 has been designated *calB*; presumably RB111 is a triple mutant having defects at *calA*, *calB*, and *corA*. RB111 and all of its derivatives harboring the three mutations form very small colonies on minimal plates but grow sufficiently well in liquid medium for the preparation of membrane vesicles.

TABLE 2. *Growth properties of wild-type and calcium-sensitive mutants*

Strains	Relevant genotype[a]	Growth properties[b]				
		7.8[c]			5.6[c]	
		0[d]	25[d]	50[d]	0[d]	50[d]
AW574 and RB202	Wild type	+	+	+	+	+
2561	*calA corA*	+	−	−	+	+
RB165	*calA*	+	+	+	+	+
RB146	*corA*	+	+	−	+	+
MP2	*corA*	+	+	−	+	+
RB111	*calA calB corA*	+	−	−	+	−
RB205	*calA calB*	+	+	+	+	+
RB063	*calC*	+	±	−	+	+
RB073	*calD*	+	±	−	+	+

[a] The presence of the *calA* defect in RB165 was inferred from $^{45}Ca^{2+}$ uptake properties of everted vesicles prepared from this strain, as shown in Fig. 3. The presence of *calA* and *calB* defects in strains RB205 was inferred from a genetic study in which P1 phage grown on RB205 was unable to donate *cal*$^+$ to RB160; transductants with the CalB phenotype could be recovered from this transduction.

[b] Colony-forming ability was determined by streaking for single colony isolation on either medium E (pH 7.8) or medium H (pH 5.6). Symbols: +, observable colonies after 24-h incubation at 37°C; −, no observable growth even after 72 h at 37°C; ±, small colonies after 48 h at 37°C.

[c] Medium pH.

[d] $CaCl_2$ (mM).

Transport in everted membrane vesicles of calcium-sensitive mutants. Figure 1 shows $^{45}Ca^{2+}$ uptake in everted vesicles prepared from three mutant strains compared with transport in vesicles from their parents. Surprisingly, strain 2561, which is sensitive to 25 mM $CaCl_2$, showed consistently higher levels of uptake than its parent strain AW574; both the initial rate of calcium entry and the final level attained seemed to be affected. In many different preparations, strain 2561 yielded two- to threefold the level of calcium uptake as AW574. On the other hand, everted vesicles from both RB063 and RB073 displayed reduced levels of $^{45}Ca^{2+}$ uptake when compared with vesicles prepared from their parent. The amount of $^{45}Ca^{2+}$ taken up by any vesicle preparation varied from assay to assay or from preparation to preparation, but the relative amounts of $^{45}Ca^{2+}$ taken up by the mutants was always consistent with the data as reported in Fig. 1. The transport defects observed in these strains appeared specific for calcium, since other transport systems were unaffected; the uptake of lysine and glutamate were equivalent in whole cells of these mutants when grown under identical conditions (data not shown).

The ability of the mutant strains to form a transmembrane pH gradient, as judged by the energy-dependent quenching of quinacrine fluorescence, was unimpaired, as was their ability to oxidize D-lactate (data not shown). When the Ca^{2+}/H^+ activity of the mutants was compared to that of the parental strain using the quinacrine fluorescence assay, equivocal results were

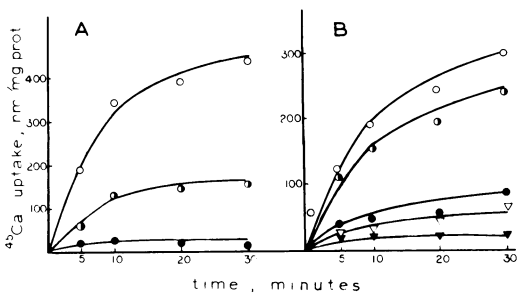

FIG. 1. *Uptake of $^{45}Ca^{2+}$ by everted membrane vesicles from mutant and wild-type strains. Transport assays were performed as described in the text using 5 mM NADH as energy source. Symbols: (A) Strain 2561 (calA corA), ○; AW574 (cal$^+$ cor$^+$), ◑; AW574 without NADH, ●. (B) RB063 (calC), ●; RB073 (calD), ▽; RB202 (cal$^+$), ○; KBT001 (cal$^+$), ◑; KBT001 without NADH, ▼.*

obtained. None of the mutants exhibited an altered affinity for Ca^{2+}. Quantitation of the quinacrine assay in terms of V_{max} values is not reliable, so, although some changes in this parameter were observed, their significance is not clear.

Mapping the calcium-sensitive lesions in strain 2561. Interrupted mating could not be done with strain 2561 due to the large numbers of spontaneous calcium-resistant colonies arising on plates containing either 25 mM or 50 mM $CaCl_2$. Thus, a series of crosses was performed using Hfr strains with various points of origins and derivatives of strain 2561 bearing suitable

auxotrophic markers. Neither Hfr Cavalli, Hfr Hayes, KL96, KL983, nor KL16 could donate calcium resistance to 2561. On the other hand, both Ra-2 and KL209 were found to be effective donors. Ra-2, having its origin at min 86.5 of the revised *E. coli* map, donates markers clockwise, whereas KL209, having its origin inserted in the *malB* gene, donated markers counterclockwise (15). This suggested that calcium sensitivity was localized between the origins of these two Hfr strains.

Further analysis using calcium sensitivity as an unselected marker and using derivatives of strain 2561 showed that calcium resistance appeared to be localized at two distinct loci of the map: one locus at about min 85 and another at min 97 (Fig. 2). That strain 2561 has no calcium-sensitive lesion mapping between the origins of KL209 and Ra-2 was verified by transduction with markers in the region. Using suitable auxotrophic derivatives of strain 2561, it was found that calcium resistance could not be cotransduced with either *metB*, *argH*, *netA*, *purD*, *malB*, or *melA*.

Because strain 2561 appeared to have a lesion mapping at about min 85, it seemed reasonable that the defect might be identical with *corA*. P1 grown on wild-type cells could transduce RB121 to *metE*$^+$ and calcium resistance (Table 3). The close linkage with *metE* suggested that this marker is indeed identical with the *corA* locus described by Park et al. (23). Supporting this, *metE*$^+$ transductants of RB121 were all still sensitive to 50 mM $CaCl_2$ when a known *corA* mutant (MP2) was used as a donor. In addition, strain 2561 is defective in Co^{2+} transport characteristic of *corA* strains (J. E. Lusk, personal communication).

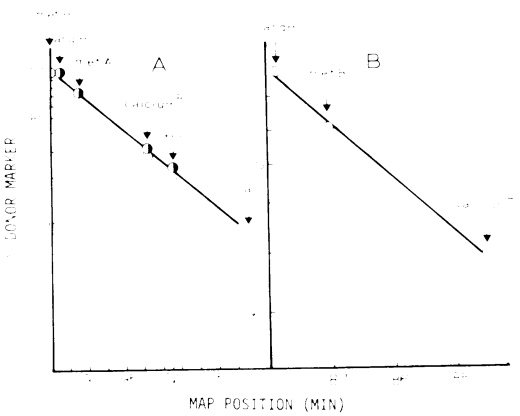

FIG. 2. *Unselected marker analysis of calcium sensitivity in strain 2561 (calA corA). Several auxotrophic derivatives of strain 2561 (RB105 and RB134) were mated with Ra2 (cal*$^+$ *cor*$^+$*) or KL209 (cal*$^+$ *cor*$^+$*). Recombinants selected for a proximal marker (metB or argH) were scored for distal markers, and calcium resistance was determined on plates containing 25 mM* $CaCl_2$*. Symbols: (A) Ra2 × RB134, ◑; Ra2 × RB105, ○. (B) KL209 × RB105, ○.*

TABLE 3. *Strain 2561 has a corA defect*

Donor (genotype)	*metE*$^+$ analyzed[a] (no. scored)	Distribution of unselected markers	
		Marker	No.
MA96 (*metE*$^+$ *corA*$^+$ *ilv*)	498	*ilv*$^+$ *cor*$^+$	161
		ilv$^+$ *corA*	106
		ilv cor$^+$	118
		ilv corA	63
MP2 (*corA ilv*$^+$ *metE*$^+$)	187	*ilv*$^+$ *corA*	187
		ilv$^+$ *cor*$^+$	0

[a] The recipient was RB121 (*corA calA metE ilv*$^+$). *metE*$^+$ transductants were analyzed for calcium sensitivity by replica plating to medium E plates containing 50 mM $CaCl_2$.

***corA* mutation and calcium transport.** To determine whether the *corA* defect itself was responsible for the elevated levels of calcium transport in strain 2561, a series of strains was constructed having various combinations of *corA* and *calA* mutations, where the mutation in strain 2561 which maps at min 97 was designated *calA*. Two isolates of RB146 were derived from a mating between RA-2 and RB134 and were shown to harbor the original *corA* mutation by their inability to transduce RB121 to *metE*$^+$ *cor*$^+$. These strains were resistant to 25 mM $CaCl_2$ but were still sensitive to 50 mM $CaCl_2$, a phenotype similar to *corA* mutants. The separate isolates of RB146 exhibited levels of calcium uptake equivalent to that in vesicles from the wild-type strain AW574 (Fig. 3A). In contrast, *cor*$^+$ *metE*$^+$ transductants of RB121 still showed the increased levels of calcium uptake characteristic of strain 2561 (Fig. 3B). Even though the *calA* defect is still present in RB165, and altered transport activity is observed, it is completely resistant to $CaCl_2$ concentration up to 75 mM. We have been unable to find a growth phenotype for *calA* mutants outside a *corA* genetic background. Thus, the *corA* gene appears to have no role in $^{45}Ca^{2+}$ uptake in everted vesicles, as measured by our assay. The *calA* gene defect results in higher levels of calcium uptake in everted vesicles and in increased sensitivity to $CaCl_2$ in a *corA* genetic background.

Localization of the *calA* gene defect. A mating was performed between RB160 and KLF17/KL32, a diploid F′ strain bearing markers in the region from the origin of Hfr Hayes through *purA* (14). Of 360 *purA*$^+$ excon-

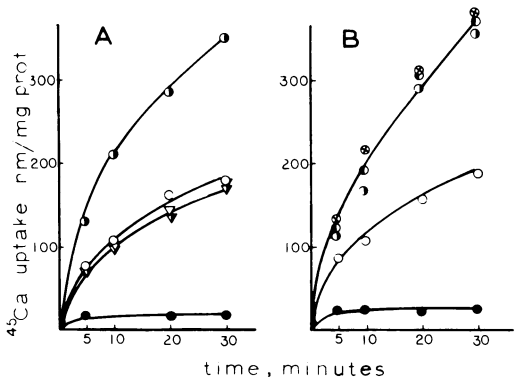

FIG. 3. *Uptake of $^{45}Ca^{2+}$ by everted membrane vesicles from calA cor$^+$ and cal$^+$ corA strains. Transport assays were performed as described in the text using 5 mM NADH as energy source. Symbols: (A) RB134 (calA corA), ◑; two separate isolates of RB146 (calA cor$^+$), ▽ and ▼; AW574 (cal$^+$ cor$^+$), ○; AW574 lacking NADH, ●. (B) Three separate isolates of RB165 (calA cor$^+$), ⊗, ◑, and ◐; AW574, ○; AW574 lacking NADH, ●.*

jugants from such a mating, only 14 were still sensitive to 25 mM $CaCl_2$. All of these were still sensitive to 50 mM $CaCl_2$, and thus are still *corA*. KLF19/KL132, an F′ covering *pyrB* but not *purA*, was not able to donate calcium resistance to RB160. *calA* was localized further by three-point transduction (Table 4). In five separate experiments using χ316 (*cycA purA$^+$ pyrB$^+$ calA$^+$*) as the donor, the data indicated a close linkage of *calA* with *purA* and *cycA*, with the order most probably being *purA-calA-cycA* (Fig. 4). In some experiments from Table 4, *cal$^+$* was scored by the ability of replicate colonies to form background growth on minimal plates with succinate as the carbon source, with threonine and histidine as the required amino acids. When *pyrB$^+$* was the selected marker, 96% of the transductants did not form this background and were scored as *calA*. Only 4% of the *pyrB$^+$* transductants did not form the background growth, and all of those were also resistant to 25 mM $CaCl_2$. All of these were also *purA* (Table 4). In addition, when strain 2561 served as donor and *purA$^+$* was selected, none of the transductants was resistant to 25 mM $CaCl_2$. The *calA* gene is probably not the *corB* described by Park et al. (23) in their study of Mg^{2+} transport mutants, since strain 2561 is still sensitive to 0.1 mM Co^{2+} when tested on plates having trace amounts of Mg^{2+}. Also, the *corB* locus is more tightly linked with *pyrB* than is the *calA* locus (23). A *corA corB* double mutant also displays sensitivity to 25 mM $CaCl_2$ (J. E. Lusk, personal communication).

***calB* mutation and calcium transport.** Figure 5 shows calcium uptake properties of everted vesicles prepared from RB107 (a *metB* derivative of RB111) compared with vesicles from strain 2561 and AW574. Although the levels of $^{45}Ca^{2+}$ uptake shown in this experiment are lower than those usually obtained, the pattern is a reproducible one, with RB107 always showing inability to transport $^{45}Ca^{2+}$. Addition of 5 μM

TABLE 4. *Transduction data for the calA gene*

Donor[a,b] (genotype)	Selected marker (no. scored)	Distribution of unselected markers	
		Marker	No.
χ316 (*cycA purA$^+$ pyrB$^+$ calA$^+$*)	*purA$^+$* (2,129)	*cycA calA*	104
		cycA calA$^+$	962
		cycA$^+$ calA	186
		cycA$^+$ calA$^+$	877
Strain 7 (wild type)	*pyrB$^+$*[c] (305)	*purA calA*	294
		purA calA$^+$	0
		purA$^+$ calA	0
		purA$^+$ calA$^+$	11
2561 (*calA pyrB$^+$ purA$^+$*)	*purA$^+$* (161)	*pyrB$^+$ calA*	3
		pyrB calA	158

[a] RB160 (*cycA$^+$ purA pyrB calA*) was the recipient in each cross.

[b] When χ316 was the donor strain, five separate transductions were performed using RB160 as the recipient. *purA$^+$* transductants were selected on minimal plates with glycerol as the carbon source. Usually, calcium resistance was determined by streaking transductants on medium E plates, pH 7.8, containing 25 mM $CaCl_2$; in some experiments *calA$^+$* was scored by ability to form significant growth above background on minimal plates containing succinate as the carbon source, and threonine and histidine as the required amino acids. *pyrB$^+$* clones, because they occurred at a frequency of less than 3%, were not included in the analysis (see text).

[c] *pyrB$^+$* was selected on minimal plates containing succinate as the carbon source due to the large background observed when glycerol was the carbon source.

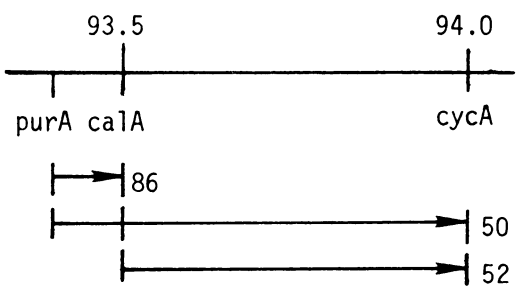

FIG. 4. *Chromosomal location of calA. See Table 4 for details.*

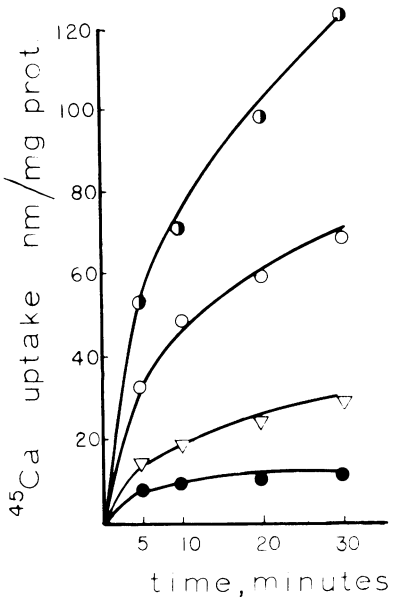

FIG. 5. *Uptake of $^{45}Ca^{2+}$ by everted membrane vesicles from a calB strain. Transport assays were performed as described in the text using 5 mM NADH as energy source. Symbols: RB107 (calA calB corA), ▽; strain 2561 (calA corA), ◑; AW574 (cal^+ cor^+), ○; AW574 lacking NADH, ●.*

A23187, which catalyzes the electroneutral exchange of protons for calcium (11), restored $^{45}C^{2+}$ transport capability to vesicles of RB107 (data not shown). This indicates that these vesicles were still capable of generating a sufficient pH gradient by the oxidation of NADH.

As shown above, the *corA* allele is necessary to express the calA phenotype, a sensitivity to 25 mM $CaCl_2$. This also appears to be the case with the CalB phenotype. When cor^+ recombinants were obtained by mating KL209 with RB107, none of the recombinants showed any sensitivity to $CaCl_2$. In other experiments, the cor^+ *calA calB* strain RB205 still showed impaired transport equivalent to RB111 (data not shown).

Mapping the *calB* gene. A close linkage of the *calB* gene with the *calA* gene was indicated: of 108 $argH^+$ exconjugates selected in a mating between Ra-2 and RB133 (*metA argH calA calB corA rpsL*), 85 were resistant to 50 mM $CaCl_2$ at pH 5.6, and 77 were resistant to 25 mM $CaCl_2$ at pH 7.8. None of the ones resistant to calcium at pH 7.8 was also sensitive at pH 5.6, whereas eight that were resistant at pH 5.6 remained sensitive at pH 7.8. This fact is taken to mean that *calB* is closely linked to, yet separate from, *calA*. It is also possible that the CalB phenotype could be expressed only in the presence of a *calA* defect. In addition, F-primes which cover the region between *purA* and *pyrB* were able to complement the *calB* defect (Table 5). F'117 was able to donate resistance to calcium at pH 5.6 to RB158, a *recA pyrB* derivative of RB111; F'119 was not. This complementation was observed both in liquid media and on plates; the reason for the poor complementation by F'117 is not known.

For unknown reasons it was impossible either to grow phage P1*kc* on derivatives of RB111 or to transduce them. To demonstrate linkage of *calB* with *purA*, P1*kc* grown on RB205, a cor^+ derivative of RB111, was used to infect RB160. In several experiments it was possible to recover poorly growing $purA^+$ transductants. These turned out to be sensitive to calcium at pH 5.6. These recombinants occurred at a frequency of 15 to 40% (39/252, 25/100, and 73/187). No such colonies were recovered when strain 2561 was used as the donor. These experiments could not establish an unequivocal gene order, but a cotransduction frequency of 15 to 40% with *purA* would tend to localize *calB* further from *purA* than *calA*. It is likely that *calB* represents a separate gene product, but adequate complementation experiments must be done to verify this.

Mapping of the *calC* gene. Unselected marker analysis using various Hfr strains mated with RB063 indicated that the *calC* gene was located near *trp*. KL208, which has its origin near the *trp* locus and donates markers counterclockwise, was found to be the most efficient donor. Of 108 $aroA^+$ colonies selected from a mating of KL208 with RB214, 104 were calcium resistant. Of 144 trp^+ recombinants selected using KLF26/KL181 as donor, 77 were resistant to 50 mM $CaCl_2$. Likewise, F152/KL253 was able to donate both calcium resistance and $galE^+$ to RB228. A tight linkage of *calC* with *galE* was suggested. A very low cotransduction frequency of *calC* with *galE* was observed (<1%) (Table

TABLE 5. *Complementation of calB*

Strain[a]	Doubling time (h)[b]	
	Control	+50 mM $CaCl_2$
RB158	4.6	>48
F'119/RB158	3.4	>48
F'117/RB158	2.3	6.7

[a] $pyrB^+$ diploids were constructed from KLF17/KL132 and KLF19/K1132 as described in the text. In each instance the presence of the episome was verified by the ability to donate $pyrB^+$ to a *pyrB* F⁻ strain.

[b] Medium H, pH 5.6, supplement with glycerol was used for growth assays.

6). The data from a series of transductional crosses indicated a close linkage of *calC* with *nagA* and *kdp* (Table 6). Analysis of the frequencies of recombinant classes obtained indicated that the most probable gene order is *nagA-calC-kdp-gltA-galE* (Fig. 6). In experiment 5 of Table 6, *nagA* was selected since results from experiment 4 could not distinguish between the order *calC-nagA-kdp* or *nagA-calC-kdp*. In addition to the *calC* mutation in RB063, three independently isolated calcium-sensitive mutants also cotransduced with *kdp* and showed transport defects (data not shown). None of these other mutants was able to donate calcium resistance to RB252. Whether or not these mutations are in the same gene is not known.

Mapping the *calD* locus. As shown in Fig. 2, RB073 (*calD*) showed decreased ability to transport $^{45}Ca^{2+}$. Preliminary mapping experiments indicated that the mutation in RB073 was distinct from the *calC* locus. In further experiments, a close linkage with *proC* was suggested. The results of a three-point transduction using either *lac* or *proC* as the selected markers indicated that the gene order is *lac-proC-calD* (Table 7).

DISCUSSION

The data presented in this paper indicate the existence of several components comprising the calcium/proton antiport system of *E. coli*. We have shown that there are at least four separate mutations which result in alterations in $^{45}Ca^{2+}$ transport activity in everted vesicles. Mutations at either the *calC* or the *calD* locus lead to impaired ability to accumulate Ca^{2+}. Apparently these mutations specifically affect calcium transport, since other transport systems measured showed no defect. A mutation at the *calA* locus

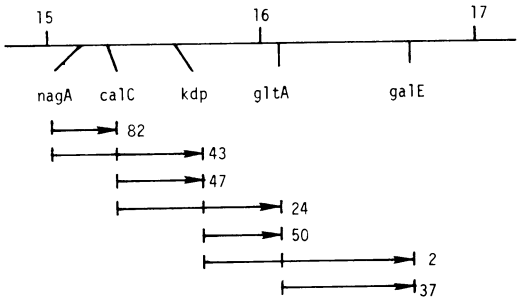

FIG. 6. *Chromosomal location of calC. nagA was scored or selected on minimal plates containing 0.2% N-acetylglucosamine. kdp was scored or selected on low-potassium plates as described by Epstein and Davies (10). gltA$^+$ was selected on minimal plates with glycerol. The cotransduction frequency of kdp with gltA was determined in an experiment in which the proportion of kdp$^-$ colonies among gltA$^+$ transductants was examined using FRAG5 as donor and G19 as recipient. Other values are average cotransduction frequencies from the data given in Table 6.*

TABLE 6. *Transduction of calC*

Donor (relevant genotype)	Recipient (relevant genotype)	Selected marker[a] (no. scored)	Distribution of unselected markers	
			Marker	No.
PL2 (*gltA$^+$ calC$^+$ galE*)	RB241 (*gltA calC gal$^+$*)	*glTA$^+$* (748)	*gal$^+$ cal$^+$*	184
			gal$^+$ calC	536
			galE cal$^+$	0
			galE calC	28
Hfr Hayes (*gal$^+$ cal$^+$*)	RB228 (*galE calC*)	*galE$^+$* (187)	*gal$^+$ cal$^+$*	1
			gal$^+$ calC	186
PL2 (*kdp$^+$ cal$^+$ galE*)	RB252 (*kdpABC5 calC gal$^+$*)	*kdp$^+$* (152)	*cal$^+$ gal$^+$*	60
			cal$^+$ galE	0
			calC gal$^+$	88
			calC galE	4
TK2240 (*nagA kdp$^+$ cal$^+$*)	RB252 (*kdpABC5 calC gal$^+$*)	*kdp$^+$* (1,112)	*nag$^+$ cal$^+$*	66
			nag$^+$ calC	368
			nagA cal$^+$	435
			nagA calC	66
RB252 (*kdpABC5 calC nagA$^+$*)	RB257 (*kdp$^+$ cal$^+$ nagA*)	*nagA$^+$* (561)	*kdp$^+$ cal$^+$*	85
			kdp$^+$ calC	244
			kdp cal$^+$	15
			kdp calC	217

[a] *nagA* was scored on minimal plates containing 0.2% *N*-acetylglucosamine. *kdp* was scored on K/0 plates as described by Epstein and Davies (10). *gltA* was scored on minimal plates with glycerol as the carbon source.

TABLE 7. *Transduction data for the calD gene*

Donor (genotype)	Recipient (genotype)	Selected marker (no. scored)	Distribution of unselected markers[a]	
			Marker	No.
Strain 7 (*cal*$^+$ *pro*$^+$ *lac*$^+$)	RB073 (*calD proC lac*)	*proC*$^+$ (411)	*lac*$^+$ *cal*$^+$	7
			lac$^+$ *calD*	45
			lac cal$^+$	96
			lac calD	263
Strain 7	RB073	*lac*$^+$ (112)	*cal*$^+$ *proC*$^+$	2
			cal$^+$ *proC*	1
			calD proC$^+$	14
			calD proC	95

[a] Calcium sensitivity of transductants was determined by replicate plating to medium E plates containing 50 mM $CaCl_2$.

was shown to result in a two- to threefold increase in the amount of $^{45}Ca^{2+}$ taken up in everted vesicles prepared from a *calA* mutant. The presence of another mutation, *calB*, in the presence of *calA*, also resulted in impaired ability to concentrate $^{45}Ca^{2+}$. All of the *cal* mutations result in calcium sensitivity in whole cells. However, the CalA and CalB phenotypes are expressed as calcium sensitivity only in a *corA* genetic background. Introduction of the *cor*$^+$ allele by transduction or mating restores wild-type response to calcium ions, but the transport defects can still be measured. We have defined map locations for *calA*, *calC*, and *calD*; *calB* has not been localized as accurately but maps close to but separately from *calA*. It also appears that the *corA* mutation, although it results in calcium sensitivity, has no role in $^{45}Ca^{2+}$ uptake by everted vesicles. The reason for the calcium sensitivity of *corA* strains is not known, but it is possible that calcium ion is able to repress the remaining Mg^{2+} transport system, thus effectively starving cells for Mg^{2+} (17, 21). The concentration of Ca^{2+} inside the cell is low even when high concentrations of calcium are present in the growth medium, suggesting that calcium may be able to repress Mg^{2+} transport without entering the cell. We have found that Mg^{2+} can partially reverse calcium sensitivity of a *corA* strain but not a *corA calA* strain. The fact that the Mg^{2+} can act as an allosteric inhibitor of the Ca^{2+}/H^+ system, as judged by the quinacrine fluorescence assay, argues for some role for internal cell Mg^{2+} in the activity of the Ca^{2+}/H^+ system: inhibition occurs at physiological levels of Mg^{2+} (7). However, Ca^{2+} does not inhibit Mg^{2+} transport in whole cells, although some inhibition of Co^{2+} transport by Ca^{2+} has been reported (20). Likewise, Ca^{2+} does not inhibit Mn^{2+} transport via the high-affinity Mn^{2+} transport system (29). In our system, no differences in the patterns of Mg^{2+} inhibition of the Ca^{2+}/H^+ system were observed between strains 2561 (*calA corA*) and AW574 (wild type). If the $^{45}Ca^{2+}$ uptake properties of everted vesicles from strain 2561 reflect an increased rate of efflux from whole cells, it is difficult to see how this can lead to increased sensitivity to $CaCl_2$. It would seem that the intracellular concentrations of ions are very tightly regulated, but the exact relationships between a system designed primarily to extrude an ion, the Ca^{2+}/H^+ system, and systems such as the magnesium transport system, which are responsible for active accumulation of an ion into whole cells, remain unclear.

Why there is no obvious difference between mutant and wild-type vesicles in Ca^{2+}/H^+ activity as measured with the quinacrine fluorescence assay is also unclear. However, the assay used for the uptake of $^{45}Ca^{2+}$ is very sensitive to small changes in Ca^{2+} concentration, both externally and internally: since 5 to 10 mM phosphate is present in all of these assays, a *meta*-stable state exists such that a small change in the amount of Ca^{2+} taken up could lead to a large difference in the amount of calcium phosphate salts precipitated intravesicularly. The assay based on quinacrine fluorescence is indirect, but, in principle, can measure calcium fluxes across the membrane in both directions. Based on the nature of the steady-state level of quinacrine fluorescence after addition of calcium, we have postulated that everted vesicles are relatively leaky to Ca^{2+} (7). There could be a system for calcium uptake into whole cells which responds electrophoretically to the membrane potential. Perhaps the *calA* mutation represents a lesion in such a system. It may be that both assays measure the same activity and that differences in the quinacrine assay might only be observed when appropriate mutations (i.e., double mutants) are introduced. Several such strains are available, but are

inadequate for several reasons: one strain, RB265, a *calC calD* double mutant obtained by construction, grows extremely poorly on all media tested, even when the calcium chelator ethyleneglycol-bis(β-aminoethyl ether)-*N,N*-tetraacetic acid is present; the other doubly mutant strains, RB111 or RB205, for unknown reasons quench quinacrine very poorly.

The nature of the regulation of the Ca^{2+}/H^+ system poses some interesting problems: it is apparently not repressed or induced by concentrations of up to 20 mM $CaCl_2$ in the medium. It is possible, on the other hand, that there is a role of the pH of the medium in regulation: some data exist suggesting that the *calB* gene product is present only after growth at pH 5.6.

The physiological functions of the Ca^{2+}/H^+ system are unknown. The most obvious function would be to lower intracellular Ca^{2+} concentrations. We have postulated that Ca^{2+} is partly responsible for regulating chemotactic responses (7, 26). Ordal has recently shown that, in the presence of ionophore A23187, Ca^{2+} causes tumbling in *Bacillus subtillis* (22). We have made similar observations in Tris-EDTA-treated *E. coli* (unpublished results). None of the mutants we have isolated, however, show any obvious chemotactic defects. The *calA calB* double mutant RB205 appears to show some subtle variations in chemotactic response: when external calcium is lowered by the addition of ethyleneglycol-bis(β-aminoethyl ether)-*N,N*-tetraacetic acid, RB205 cannot be caused to tumble by the addition of a repellant; this is not true for strain 2561 or for the wild type (unpublished results).

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